DISEASE RESISTANT TRANSGENIC PLANTS

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to methods and materials for conferring disease resistance to plants. More particularly, the invention relates to transgenic plants containing a heterologous nucleic acid which confers disease resistance, particularly against infectious pathogens, such as viruses. The invention further relates to methods and materials for preparation of such transgenic plants.

Infectious diseases of cultivated plants cause substantial reductions in food, forage and fiber throughout the world. Control of these diseases has been based primarily on cultural practices that include removal of infected debris, eradication of weed hosts (herbicide applications), prevention of vector transmission (pesticide applications), indexing for pathogen-free starting material (seed or vegetative propagules) and breeding for disease resistance. Large scale methods for curing plants once they have become infected with viruses do not exist. Thus, the control of diseases is dependent upon methods to prevent or delay the establishment of infection.

Of the above disease control measures, breeding for resistance is generally one of the most economical and practical methods, as it requires no additional labor or expense to the grower. Moreover, controlling diseases with resistance does not require applications of herbicides or pesticides to eliminate weed hosts and insect vectors. Thus, host resistance is one of the most environmentally safe and durable methods for controlling plant diseases. Unfortunately, in many plant-virus systems, resistance is not available and cannot be obtained using traditional plant-breeding strategies. However, recent advances in molecular biology and gene manipulation have proven helpful in integrating new disease resistance factors into plant-virus systems where none existed before.

Background Art

The development of transgenic plants has proven to be a valuable strategy for protecting plants from viral diseases. For example, Stubbs, G. et al., United States patent 5,723,750 describe transgenic plants expressing genes encoding wild-type and modified coat proteins of different virus groups. These transgenic plants have been shown to have varying levels of resistance to infection by the corresponding virus. Unfortunately, the expression of heterologous genes encoding coat proteins does not confer broad resistance to viral infections and has no effect on pathogenesis caused by other infectious agents.

Gene-for-gene disease resistance is an important plant defense mechanism against pathogens. It is induced only when the host plant carrying a resistance (R) gene is challenged by a pathogen carrying a matching avirulence (Avr) gene (Keen, 1990). (A bibliography is provided at the end of the written description.) The specific interaction between a

matching pair of R-Avr genes usually induces the hypersensitive response ("HR") and results in the local containment of the invading pathogen at sites of attempted ingress. During the HR, the recognition of a pathogen induces a rapid cell death process that results in the formation of a zone of dead cells (necrosis) around the site of infection. This HR lesion is believed to inhibit further spread of the pathogen and to generate a signal that activates host defense mechanisms and, in many cases, induces long-lasting systemic resistance to a broad spectrum of pathogens (Ross, 1961; Ryals et al., 1996; Sticher et al., 1997). Induction of such systemic resistance is termed systemic acquired resistance ("SAR") and is accompanied by an oxidative burst, an increase in the rate of synthesis of several pathogenesis-related ("PR") proteins, alterations in cell-wall structure, and the accumulation of salicylic acid ("SA") (Malamy et al., 1990; Metraux et al., 1990; Ward et al., 1991; Hammond-Kosack and Jones, 1996; Lamb and Dixon, 1997; Yang et al., 1997).

Methods for inducing the HR in infected plants have been described. For example, Lam, E. et al., U.S. patent 5,629,470, have described a process for providing higher plants with enhanced resistance to pathogenic attack by one or more plant pathogens by transforming cells of the plants with the bacterio-opsin (bO) gene.

More than a dozen R genes specific for bacteria, viruses, fungi or nematodes have been cloned from a variety of plant species and it is striking to note that these R genes often encode structurally similar proteins, suggesting a high degree of mechanistic conservation among the pathways that plants use to

trigger defense responses (Baker et al., 1997; Hammond-Kosack and Jones, 1997).

The Avr genes of pathogens by definition encode or produce signal molecules that can initiate HR in the corresponding resistant plants. For example, Avr4 and Avr9 from the extracellular growing fungal pathogen *Cladosporium sulvum* encode pre-proproteins that are processed into small secreted peptides that can elicit R gene-dependent defense responses even in the absence of pathogens (Knogge, 1996). Similarly, the bacterial AvrD locus of *Pseudomonas syringae* pv. glycinea encodes enzymes involved in the synthesis of exported syringolide elicitors which, when injected into the intercellular leaf spaces, can elicit HR in soybean cultivars that carry the Rpg4 gene (Leach and White, 1996). However, for most of the bacterial Avr genes cloned, the Avr protein itself is the elicitor (Leach and White, 1996). These Avr genes generally encode hydrophilic proteins that lack signal sequences and do not induce HR when injected into leaves of plants with the matching R genes. It has recently been demonstrated that recognition of several bacterial Avr gene products by their matching R gene products occurs inside plant cells (Tang et al., 1996; Scofield et al., 1996; Leister et al., 1996; Gopalan et al., 1996; Van den Ackerveken et al., 1996). The import of the Avr proteins into plant cells may be through the type III secretory system encoded by the bacterial Hrp gene cluster required for HR induction and pathogenesis (Pirhonen et al., 1996; Lindgren, 1997).

Plant viruses enter cells through existing wounds and replicate intracellularly. Therefore, it is likely that viral Avr-R recognition also occurs inside plant cells. Most plant viruses have small genomes that encode genes required for replication, movement and

encapsidation. All three major types of viral genes encoding the coat protein (Bendahmane et al., 1995; Berzal-Herranz et al., 1995; Taraporewala and Culver, 1996), RNA replicase (Meshi et al., 1988; Padgett et al., 1997; Kim and Palukaitis, 1997), or movement protein (Meshi et al., 1989; Weber et al., 1993) have been demonstrated to be avirulence determinants. The 126K replicase protein (Padgett et al., 1997) of tobacco mosaic virus (TMV) is the only viral Avr protein for which the cloning of a matching R gene, N, has been reported (Whitham et al., 1994). The N gene product, predicted as a cytoplasmic protein, belongs to the NBS-LRR family of resistance genes (Baker et al., 1997).

It has been reported recently that systemic leaves of nepovirus- and caulimovirus-infected plants exhibit a strong virus resistance similar to post-transcriptional gene silencing ("PTGS"), thus providing evidence for a second type of natural defense mechanism against viruses in plants (Covey et al., 1997; Ratcliff et al., 1997). PTGS was first discovered in transgenic plants and involves the degradation of RNA species that are similar to the transcribed part of a silencer transgene. As a result, only a low level of the transgene RNA exists even if it is transcribed at a high level (Depicker and Van Montagu, 1997). PTGS confers virus resistance in transgenic plants if the silencer transgene is derived from or shares sequence homology with the genome of a plant virus (Lindbo et al., 1993; Guo and Garcia, 1997; Ruiz et al., 1998).

In support of PTGS as a natural plant defense against viruses, two proteins encoded by plant RNA viruses were identified as suppressors of PTGS in transgenic plants (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998). The HC

Pro encoded by potato virus Y blocks the maintenance of PTGS in tissues where silencing has already been established, whereas the 2b protein encoded by cucumber mosaic virus (Cmv2b) prevents the initiation of gene silencing at the growing points of the plants (Brigneti et al., 1998). Both viral proteins have been previously shown to be important for virulence determination and systemic spread (Cronin et al., 1995; Ding et al., 1995). Thus, plant viruses possessing a PTGS suppressor function provide an active and effective strategy to combat innate host resistance mechanisms. This strategy is distinct from the one employed by many plant pathogens to overcome the gene-for-gene disease resistance mechanism which is to accumulate mutations in Avr genes that can escape recognition by corresponding host R genes.

In view of the enormous economic impact of infectious diseases on agricultural production, a need continues to exist for transgenic plants having generalized resistance to pathogenic infections.

SUMMARY OF THE INVENTION

In accordance with the present invention, transgenic plants that have been stably transformed with the 2b gene of cucumovirus or an active fragment thereof have been found to possess systemic resistance to pathogenic infectious agents, such as viruses. The protein encoded by this gene activates strong disease resistance responses in host plants.

A further aspect of the invention relates to seeds and propagating parts of transgenic plants stably transformed with a cucumovirus 2b gene or an active fragment thereof. The invention further provides methods and vectors for introducing the cucumovirus 2b gene into plants.

A further aspect of the invention relates to a plant RNA virus-encoded suppressor of PTGS, which is targeted as an Avr factor by a strong host resistance mechanism resembling the gene-for-gene disease resistance. A molecular dissection of the suppressor indicates a two-domain structure in which one domain is sufficient for resistance activation, while the other was required for triggering hypersensitive cell death. This two-domain structure makes possible a novel class of disease resistance genes comprising disarmed Avr genes, i.e., those without an active cell death induction domain. Disarmed Avr genes can be obtained by such means as domain swapping to link an active resistance domain to an inactive cell death domain, or selective deactivation of an Avr cell death domain. A single such disarmed Avr gene can confer to a transgenic plant broad-spectrum pathogen resistance.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 illustrates the structural features and genome organization of the chimeric viral RNA transcripts from plasmids pTMV-t2b and pPVX-t2b.

Figure 2 is a Northern blot hybridization showing the effect of the 2b gene on expression of the pathogenesis-related protein in leaves of tobacco plants infected with tobacco mosaic virus carrying the 2b gene.

Figure 3 shows the two-domain structure of Tav2b, as revealed by domain mapping. The figure shows plant response to various 2b chimeras. In the figure, HR means observation of necrotic lesions and virus multiplication in the inoculated leaves of *N. tobaccum* cv. Samsun nn only, R means observation of virus multiplication in the inoculated leaves only, but with no visible nectrotic lesions, S means observation of susceptibility and systemic infection

Figure 4 shows the genomic organization of the TMV-30B vector and the inserted fragments from either TAV or CMV. Boxes represent open reading frames (ORF) encoded by TMV, TAV or CMV. Asterisk (*) denotes the amber stop codon which can be read through to give the 183K protein. The three sgRNA promoters are represented by arrows. Note that this vector is derived from the infectious cDNA clone of TMV-U except for the 3'-terminal sequence, including the coat protein (CP) coding sequence plus its sgRNA promoter (arrow 3), which is from TMV U5 (W.O. Dawson, pers. comm.). The 2b-coding sequences (or their mutants) from TAV (T2b) or CMV (C2b) were cloned at the PmeI site. TΔ2b1 and TΔ2b2 are derivatives of T2b with point substitutions that result in partial or complete disruption of the encoded ORF.

Figure 5 shows the accumulation of viral and plant RNAs in tobacco plants. The Samsun plants were inoculated with TMV-30B (TMV), TMV-C2b (C2b), TMV-TΔ2b2 (TΔ2b2), TMV-TΔ2b1, TMV-T2b (T2b or mock-inoculated with buffer alone (M). At different days post-inoculation (dpi) as indicated, total RNAs were extracted from either the inoculated leaves or systemic leaves and subjected to Northern blot analysis using ^{32}P-labeled DNA probes specific for TMV genomic RNA (TMV), PR-1a mRNA or the 18 S RNA (as a loading control).

Figure 6 shows the time course of the accumulation of mRNA encoded by PR-1a and PR-5 in the Samsun plants inoculated with TMV-T2b. The time (dpi) when total RNAs were extracted after inoculation is indicated above each lane.

Figure 7 shows accumulation of the six TMV recombinants int he inoculated and systemic leaves of Samsun tobacco plants. A probe for the genomic RNA of

TMV was used for the hybridization. The plant response (as abbreviated in Figure 3) to each of the recombinants is also indicated. For all six recombinants, the 2b coding sequence in the progeny virus was obtained by RT-PCR followed by DNA sequencing and found to be identical to the respective inoculum. M = mock inoculated, CT1 = TMV-CT1, CT2 = TMV-CT2, CT3 = TMV-CT3, TC1 = TMV-TC1, TC2 = TMV-TC2, TC3 = TMV-TC3.

DETAILED DESCRIPTION OF THE INVENTION

Cucumber mosaic virus (CMV) belongs to a virus genus called Cucumovirus which also includes tomato aspermy virus (TAV). Cucumovirus contains a tripartite single-stranded RNA genome that encodes five genes: 1a, 2a, 2b, 3a and coat protein. The identification and functional analysis of the 2b gene have been described in previous publications. (Ding et al., 1994; 1995; 1996). It has been demonstrated that the 2b gene encoded by the cucumoviruses is important for both systemic virus spread and virulence determination. The ribonucleotide sequence of the 2b gene is provided in SEQ ID NO. 1.

It has been discovered that when the 2b gene is expressed separately from the genome of cucumoviruses, it activates strong resistance responses in a variety of plant species upon infection with a pathogenic virus. These responses include induction of pathogenesis-related proteins and formation of necrotic lesions which eliminate the invading pathogens. Thus, in one aspect, the present invention relates to a transgenic plant stably transformed with a cucumovirus 2b gene or active fragment thereof operatively linked to a promoter that is capable of effecting expression of said gene in said plant when said plant is infected with a pathogenic organism. The cucumovirus 2b gene used for producing disease-resistant plants

advantageously is one to which the nucleic acid of SEQ ID NO. 1 will hybridize under stringent conditions.

In a related aspect, the present invention provides methods for rendering a plant resistant to disease caused by an infectious pathogenic agent, which comprises stably transforming the plant with a cucumovirus 2b gene or active fragment thereof operatively linked to a plant-active promoter that is capable of effecting expression of the gene in the plant when said plant is infected with a pathogenic organism. In a further aspect, the invention provides an expression vector comprising a cucumovirus 2b gene or active fragment thereof operably linked to a plant-active promoter.

Mutational analysis has confirmed that the 2b gene is responsible for the resistance response. Point mutations in the gene have been shown to render it non-functional and abolish the ability of the gene to activate the resistance response. In addition, it has been found that the C-terminal 26-amino acid and 45-amino acid sequences of the gene are essential for its disease-resistance function, although the codons encoding the four C-terminal amino acids can be removed without losing activity. Transfer of the codons encoding the C-terminal 26 amino acids and the C-terminal 45 amino acids of the tomato aspermy virus 2b gene to the corresponding regions of the inactive cucumber mosaic virus 2b gene does not yield an active chimeric gene; therefore, the N-terminal portion of the protein also appears to contain one or more domains that are essential for resistance activation. Accordingly, the invention relates to transgenic plants and vectors that contain an active fragment of the 2b gene.

The 2b gene also has been found to be dual functional. Depending on the virus genetic background from which it is expressed, 2b can function as either a virulence or an avirulence determinant. These findings suggest that one may search among susceptible cultivars for R-like genes that can potentially recognize virulent proteins by *in planta* expression from heterologous viral vectors. It is likely that many Avr gene products are virulence factors until a matching R gene evolves in a host cultivar. These results show that the plant gene-for-gene resistance is also effective in targeting as an Avr a virus-encoded suppressor of the plant gene silencing defense mechanism.

While there is a strong association between hypersensitive cell death and gene-for-gene disease resistance, recent studies have provided evidence that cell death is neither required nor sufficient for gene-for-gene resistance. It has been found that TMV-TC2, for example, induced strong virus resistance in the Samsun cultivar without visible hypersensitive cell death (Fig. 3). This result obtained through the molecular dissection of a viral Avr gene supports the view that cell death is not required for resistance.

Domain swapping between an avirulent 2b and its virulent homologue suggests a two-domain structure for an Avr protein. The N-terminal region of 69 amino acids of Tav2b, for example, constitutes the first domain that is essential and sufficient for resistance activation. Thus, both TMV-TC1 and TMV-TC2, encoding a complete resistance domain, induced strong virus resistance in the challenged tobacco plants. Complete (e.g., TMV-CT1, 2 and 3) or partial (e.g., TMV-TC3) replacements or deletion (e.g., TMV-TΔ2b1) of this domain result in the loss of the activity in resistance

activation. The second domain, encompassing amino acids 70-9 of Tav2b, is tentatively referred to as the "cell death" domain because its absence in TMV-TC2 leads to a loss of cell death induction, but without apparent effect on resistance activation. However, although the whole Cmv2b is inactive, it cannot be ruled out that the Cmv2b C-terminal 34 aa fused in TC2 may function as a positive or negative modulator of cell death initiation, leading to a local symptomless phenotype of TMV-TC2. Notably, these two functional domains correspond to the overlapping and non-overlapping regions of the cucumoviral 2b genes, as defined previously according to whether or not it overlaps with the 2a gene (Ding et al., 1995).

Distinct from the N-terminal resistance domain (which can act independently), the cell death domain is dependent on the presence of a functional resistance domain. For example, both TMV-CT2 and TMV-CT3 encode a complete cell death domain, but fail to induce necrotic lesions in the inoculated leaves (Fig. 3). Other evidence supporting this observation is that the two amino acid substitutions that rendered Tav2b inactive in triggering resistance and cell death are both located within the resistance domain, but outside of the cell death domain. It is interesting that the resistance domain is functionally independent in contrast to a conditional cell death domain. Thus, cell death signaling, which is usually placed downstream of the Avr-R interaction in the HR network (Hammond-Kosack and Jones, 1996; Lamb and Dixon, 1997), probably involves a subsequent interaction of the pathogen Avr product with another plant protein, possibly via its cell death domain.

It will be of practical importance to determine if the concept of the two-domain structure of Tav2b also

applies to Avr genes encoded by any other viral, bacterial and fungal pathogens. Constitutive expression of an Avr gene in a cultivar that contains the matching R gene should generate a constitutive broad-spectrum disease resistance. However, this type of resistance cannot be readily utilized because the specific Avr-R interaction also leads to immediate activation of the hypersensitive cell death (Culver and Dawson, 1991; Gopalan et al., 1996; Leister et al., 1996; Scofield et al., 1996; Tang et al., 1996; Van den Ackerveken et al., 1996; Gilbert et al., 1998). By removing the active cell death domain, swapping it with ab inactive cell death domain, or selectively deactivating the cell death domain will maintaining its macrostructural integrity, it would be possible to induce system resistance with cell death. Because the SAR, once induced, is non-specific as to pathogen (be it viral, fungal, bacterial, etc.), it would be possible to confer broad-spectrum resistance by incorporating a single disarmed Avr (preferably disarmed by domain swapping) as a transgene. This is an efficient means of providing a pathogen-resistance plant.

The 2b gene or its active fragment (hereinafter, the "2b gene"), or any Avr useful in this invention, may be introduced into plants using conventional vectors and procedures. Generally, such techniques involve inserting the gene into an expression vector which contains the necessary elements for the transcription and translation of the inserted coding sequences and one or more marker sequences to facilitate selection of transformed cells or plants.

A number of plant-active promoters are known in the art and may be used to effect expression of the nucleic acid sequences disclosed herein. Constitutive

promoters, such as the nos promoter or the 35S promoter of cauliflower mosaic virus, may be used; however, constitutive expression may be harmful to the transgenic plants. Accordingly, inducible promoters, especially pathogen-inducible promoters, such as pathogenesis-related protein promoters are preferred.

Once the gene has been cloned into an expression vector, it may be introduced into a plant cell using conventional transformation procedures. The term "plant cell" is intended to encompass any cell derived from a plant including undifferentiated tissues such as callus and suspension cultures, as well as plant seeds, pollen or plant embryos. Plant tissues suitable transformation include leaf tissues, root tissues, meristems, protoplasts, hypocotyls, cotyledons, scutellum, shoot apex, root, immature embryo, pollen, and anther.

One technique for transforming plants is by contacting tissue of such plants with an inoculum of a bacterium transformed with a vector comprising the gene in accordance with the present invention. Generally, this procedure involves inoculating the plant tissue with a suspension of bacteria and incubating the tissue for 48 to 72 hours on regeneration medium without antibiotics at 25-28° C.

Bacteria from the genus *Agrobacterium* can be utilized advantageously to transform plant cells. Suitable species of such bacteria include *Agrobacterium tumefaciens* and *Agrobacterium rhizogens*. *Agrobacterium tumefaciens* (e.g., strains LBA4404 or EHA105) is particularly useful due to its well-known ability to transform plants.

Another approach to transforming plant cells with the nucleic acid of this invention involves propelling inert or biologically active particles into plant

cells. This technique is disclosed in U.S. patents 4,945,050, 5,036,006 and 5,100,792 all to Sanford et. al., which are hereby incorporated by reference. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and to be incorporated within the interior thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles with the vector comprising the 2b gene. Biologically active particles (*e.g.*, dried yeast cells, dried bacterium or a bacteriophage, each containing DNA sought to be introduced) can also be propelled into a plant cell tissue.

Another method of transforming plant cells is the electroporation method. This method involves mixing the protoplasts and the desired DNA and forming holes in the cell membranes by electric pulse so as to introduce the DNA into the cells, thereby transforming the cells. This method currently has high reproducibility and various genes have been introduced into monocotyledons, especially rice plants by this method (Toriyama et. al., 1988, Shimamoto et al., 1989 and Rhodes et al., 1988).

Similar to the electroporation method is a method in which the desired gene and protoplasts are mixed and the mixture is treated with polyethylene glycol ("PEG"), thereby introducing the gene into the protoplasts. This method is different from the electroporation method in that PEG is used instead of an electric pulse (Zhang W. et. al., 1988, Datta et al., 1990 and Christou et al., 1991).

Other methods include 1) culturing seeds or embryos with nucleic acids (Topfer R. et al., 1989, Ledoux et al., 1974) 2) treatment of pollen tube, (Luo

et al., 1988) 3) liposome method (Caboche, 1990) and 4) the microinjection method (Neuhaus G. et al., 1987).

Known methods for regenerating plants from transformed plant cells may be used in preparing transgenic plants of the present invention. Generally, explants, callus tissues or suspension cultures can be exposed to the appropriate chemical environment (e.g., cytokinin and auxin) so the newly grown cells can differentiate and give rise to embryos which then regenerate into roots and shoots.

The genes of the present invention are useful in enhancing resistance to disease-causing pathogens in both monocotyledonous plants ("monocots")and dicotyledonous plants ("dicots"), such as corn, wheat, rice, millet, oat, barley, sorghum, sunflower, sweet potato, alfalfa, sugar beet, brassica species, tomato, pepper, soybean, tobacco, melon, squash, potato, peanut, pea, cotton or cacao.

The invention is further illustrated by the following examples, which are not intended to be limiting.

EXAMPLES

Example 1

(Vector Construction)

Several efficient *in planta* expression systems based on plant RNA viruses have been developed in recent years. The vectors based on tobacco mosaic virus (TMV) (U.S. patent 5,589,367) and potato virus X (PVX; Chapman et al., 1992) were used in this work for expressing the 2b genes of cucumoviruses. Figure 1 shows the structural features of the chimeric viruses (TMV-t2b and PVX-t2b) that were constructed. The coding

sequence of the 2b gene of TAV (SEQ ID NO. 1, encoding 95 amino acids) was prepared by PCR amplification of the TAV ORF 2b coding sequence (nucleotides 2447-2734 of RNA2) from pQCD2qt (Ding et al., 1996) using the Pfu DNA polymerase (Stratagene). This sequence was inserted into the genome of TMV and PVX upstream of the respective coat protein (CP) gene. The PCR fragment was blunt-end cloned at the *Pme*I site of a TMV vector, known as pTMV-30B, to yield TMV-t2b (Figure 1). The TAV insert in TMV-t2b was excised as an *Age*I - *Xho*I fragment (see Figure 1), and this fragment was end-filled and cloned into *Cla*I-digested and end-filled pPC2S (an expression vector based on potato virus X (Chapman et al., 1992)) to produce PVX-t2b. The 2b gene expression was controlled by independent promoters (arrows labelled as 1 and 3 in Fig. 1) which are recognized only by the respective RNA-dependent RNA polymerase encoded by TMV or PVX.

The 2b-expressing derivatives of TMV or PVX (TMV-t2b and PVX-t2b) were used to infect plants and the functional role of the 2b gene was inferred from differences in the induced plant responses between the wild type and its 2b-expressing derivative.

Example 2

(Resistance in *Nicotiana tobacum* Samsun)

TMV-t2b induced a typical hypersensitive response (HR) in Samsun (nn) tobacco plants. Plasmids pTMV-30B, pPC2S and their derivatives were linearized and transcribed *in vitro* as described (Chapman et al., 1992) in the presence of the cap analog (NEB) using the T7 RNA polymerase (Promega). The capped RNA transcripts were inoculated mechanically onto fully developed leaves of *Nicotiana tabacum* cv Samsun (nn). The plants were incubated in the Conviron growth chambers (24°C constant, 75% humidity and 16 hours light/8 hours dark). Local necrotic lesions appeared on the inoculated leaves about 4 days after inoculation and the rest of the plant was symptom-free for as long as observations were made (5 weeks). The failure of TMV-t2b to spread systemically in Samsun (nn) plants was further confirmed by Northern blot analysis which detected no accumulation of viral RNAs in upper uninoculated leaves. Furthermore, transcription of the mRNAs for pathogenesis-related (PR) protein 1 (PR-1), PR-3 and PR-5 was induced in the inoculated leaves. See Figure 2, which is a Northern blot for leaves of plants challenged with TMV-t2b or TMV. Northern blot hybridization was performed using PR-1a cDNA as a probe (obtained by PCR amplification from tobacco plants based on the sequence disclosed by Cornelissen, B.J. et al. (1987)). Total RNAs extracted from plants 5 days (lane 1), 7 days (lane 2), 10 days (lane 3), and 13 days (lane 4) showed increasing expression of PR-1. Lanes 5 and 6 were infected with wild-type TMV; however, the tobacco genotype was nn for lane 5 and NN for lane 6.

These results showed that the Samsun (nn) tobacco plants were resistant to TMV-t2b and that challenge inoculation by TMV-t2b induced the expression of both the morphological (local necrotic lesions) and molecular (PR protein induction) markers of HR in the plants.

It is known that *N. tabacum* Samsun (nn) tobacco contains no resistance gene specific to TMV, and this is confirmed in this study that when infected with the vector TMV-30B alone, the tobacco plants developed systemic mosaic symptoms and no induction of the PR genes was observed. Thus, it is concluded that the resistance responses of the tobacco plants to TMV-t2b challenge is due to the *in cis* expression of the TAV 2b gene from the TMV genome.

Example 3

(Demonstration that the 2b Gene is Responsible for Resistance)

Two mutants of TMV-t2b, each containing point mutations to disrupt the open reading frame 2b, were constructed. TMV-tΔ2b1 (SEQ ID NO. 2) is predicted not to translate any of the 2b protein in infected plants. In plants infected with TMV-tΔ2b2 (SEQ ID NO. 3), however, a truncated 2b protein missing the C-terminal 52 amino acid residues is expected to be expressed. Neither TMV-tΔ2b1 nor TMV-tΔ2b2 induced local necrotic lesions in the inoculated leaves and transcription of mRNAs for PR proteins was also not induced. Therefore, it is the TAV 2b protein that functions as the activator of resistance responses. The inserted TAV nucleotide sequence *per se* played no role in the HR elicitation. In addition, it appears that the

C-terminal 52 amino acid sequence of the TAV 2b protein is essential for this activity (see below).

Example 4

(Determination of Resistance Activation Domain)

The 2b gene encoded by the Q strain of cucumber mosaic virus (CMV) (SEQ ID NO. 4) was similarly engineered to be expressed from the TMV genome. The derivative, called TMV-q2b, systemically infected Samsun tobacco plants, did not induce necrotic lesions on the inoculated leaves, nor did it induce transcription of mRNAs for PR proteins. This shows that, in contrast to the TAV 2b protein, the CMV 2b protein was inactive in resistance activation.

To map the domain important for resistance activation, the TAV 2b protein as encoded by TMV-t2b was progressively replaced from the C-terminus by the structurally equivalent regions of the CMV 2b protein. Infectivity assays showed that replacing the C-terminal four amino acids of the TAV 2b protein retained its HR triggering activity. However, the replacement of the C-terminal 26 or 45 amino acids of the TAV 2b protein abolished its ability to trigger HR. This indicates that the C-terminal 26 amino acids of the TAV 2b protein is essential for resistance activation in tobacco plants, although the codons encoding the four C-terminal amino acids can be removed without losing activity. Transfer of the codons encoding the C-terminal 26 amino acids and the C-terminal 45 amino acids of the tomato aspermy virus 2b gene to the corresponding regions of the inactive cucumber mosaic virus 2b gene does not yield an active chimeric gene; therefore, the N-terminal portion of the protein also

appears to contain one or more domains that are essential for resistance activation.

Example 5

(Resistance in Other Plant Species)

Both *Nicotiana benthamiana* and *Physalis floridana* plants are similar to the Samsun tobacco in that they are susceptible to TMV, and the infected plants do not develop HR. Infectivity assays showed that challenge inoculation with TMV-t2b induced typical local necrotic lesions in the inoculated leaves of both *Nicotiana benthamiana* and *Physalis floridana* plants, while the uninfected parts of the plants remained symptom-free. These results suggest that the TAV 2b gene is also capable of activating resistance in these plant species against TMV. The fact that the TAV 2b gene can activate resistance against TMV in three different plant species of two genera suggests that it will function similarly in a wide range of host species.

Example 6

(Resistance Against Potato Virus X)

Both Samsun (nn) and Xanthi-nc (NN) tobacco (N. tabacum) plants are fully susceptible to potato virus X (PVX) and to the RNA transcripts from the PVX-based vector (pPC2S) (Chapman et al., 1992). However, inoculation with the PVX-t2b transcripts induced HR in leaves of both tobacco varieties. The necrotic lesions induced by PVX-t2b infection were essentially identical to those induced by TMV-t2b on the Samsun (nn) plants. In addition, Northern blot analysis showed that transcription of the PR genes was also induced in plants challenged with PVX-t2b, but not with PVX or the

PVX vector alone. Thus, the TAV 2b gene expression *in cis* from the PVX genome is also capable of triggering the resistance responses in tobacco plants containing no specific resistance gene to PVX.

TMV and PVX are distinct plant RNA viruses of different virus genera and the encoded proteins of the two viruses have minimal sequence similarities. Therefore, it is less likely that the resistance activation by the TAV 2b gene requires specific interaction with any of the proteins encoded by the two viral vectors. These results suggests a possibility that the TAV 2b gene will be able to activate resistance mechanisms against a wide range of plant pathogens.

Both the wild-type TAV (Ding et al., 1994) and the CMV/TAV chimera CMV-qt (Ding et al., 1996) encode the TAV 2b gene which is expressed at high levels in infected plants (Shi et al., 1997). It has been shown previously that all three plant species used in this work are fully susceptible to TAV and CMV-qt (Ding et al., 1996), suggesting that these plant species do not contain a resistance gene that recognizes the TAV 2b gene. This result indicates that the resistance activation activity of the TAV 2b gene is not constitutive in these plant species and may require an induction event such as infection with certain virulent pathogens (e.g., TMV and PVX). This property distinguishes the TAV 2b gene from the known avirulence genes encoded by plant pathogens.

Example 7

(Methods used for Examples 8-12)

1. Plasmid constructs

DNA manipulations and cloning were carried out using standard procedures (Sambrook et al., 1989) unless otherwise described. DNA inserts in all constructs were sequenced in two orientations prior to use.

Plasmids pTMV-T2b, pTMV-TΔ2b1, pTMV-TΔ2b2 and pTMV-C2b: Plasmid pTMV-30B (**Fig. 4**, a gift from Professor W.O. Dawson, University of Florida, USA) is a plant gene expression vector based on TMV. The coding sequence of TAV ORF 2b (nucleotides 2447-2734 of RNA2) was PCR amplified from pQCD2qt (Ding et al., 1996) using the Pfu DNA polymerase (Stratagene) and cloned at the PmeI site of pTMV-30B to yield pTMV-T2b. The plasmid pTMV-TΔ2b1 was generated from pTMV-T2b by PCR mutagenesis as previously described (Ding et al., 1995) (incorporated herein by reference). Two nucleotide substitutions (both C --> T) were introduced at positions equivalent to nucleotides 2576 and 2582 of TAV RNA2 (Moriones et al., 1991) and converted both codons 44(CAA) and 46(CGA) of ORF 2b to stop codons (TAA and TGA). The plasmid pTMV-TΔ2b2 contained the same two nucleotide mutations as in pQCD2qt2 (Ding et al., 1996) that changed the second codon of ORF 2b to a stop codon. The coding sequence of CMV ORF 2b was obtained from pSK2b (Ding et al., 1994) as a BamHI-Asp718 fragment, which was end-filled and cloned into the PmeI site of pTMV-30B to give pTMV-C2b.

Plasmids pTMV-TC1, pTMV-TC2, pTMV-TC3, pTMV-CT1, pTMV-CT2 and pTMV-CT3: The previously described mutagenesis protocol (Ding et al., 1996) (incorporated herein by reference) that involves three separate PCRs

was used with templates pQCD2(Ding et al., 1995) or pQCD2qt (Ding et al., 1996) to generate six chimaeric DNA fragments (Fig. 3). The final PCR fragments were cloned at the PmeI site of pTMV-30B to give the six plasmid constructs listed.

Plasmid pTMV-T2bC: Nucleotide substitutions, AA --> GT (equivalent to nucleotides 2508-2509 of TAV RNA 2) and CGA --> TCT (nucleotides 2529-2531 of TAV RNA 2) were introduced by PCR into the coding sequence of Tav2b as encoded by pTMV-T2b. The resultant plasmid was called pTMV-T2bC.

Plasmids pPVX-T2b and pPVX-TΔ2b2: The inserted TAV sequence in pTMV-T2b and pTMV 2TΔ2b2 (Fig. 4) was obtained as an AgeI-XhoI fragment and cloned in the PVX vector pP2C2S.

2. In vitro transcription, plant infection and Northern blot analysis

Plasmid pTMV-30B and its derivatives were linearized by PstI whereas pPVX-T2b and pPVX-TΔ2b2 were linearized by SpeI before they were transcribed *in vitro* in the presence of cap analog using TRNA polymerase (New England Biolabs). *N. tabacum* cv. Samsun (nn) plants were grown in Conviron growth chambers (22°C constant, 75% humidity and hours photoperiod). At approximately weeks old, the youngest fully expanded leaves of the plants were dusted with carborundum and inoculated with the capped RNA transcripts (transcribed from 1µg plasmid DNA template/leaf). At various times after inoculation, leaves were excised and immediately frozen in liquid nitrogen before total plant RNAs were extracted and analyzed by Northern blot analysis as previously described (Ding et al., 1995).

Induction and suppression of PTGS of the GFP transgene as well as GFP imaging in whole plant were

carried out as described previously (Brigneti et al., 1998). Briefly, GFP expressing seedlings from line 16c were infiltrated with *A. tumefaciens* carrying a binary Ti plasmid that contains a functional 35S-GFP cassette. Three weeks after infiltration when systemic PTGS of the GFP transgene was complete, RNA transcripts from pPVX-T2b or pPVX-TΔ2b2 were inoculated to the youngest fully expanded leaves of the plants.

The following DNA fragments were labeled with α-^{32}P-dCTP by random priming as described (Sambrook et al., 1989). The SphI-StuI fragment from pTMV-30B corresponds to nucleotides 445 to 1675 of the TMV genome (Goelet et al., 1982). The probes specific for the mRNA of PR-1a (Cornelissen et al., 1987), and PR-(Cornelissen et al., 1986), and for the 18S rRNA (Venkateswarlu and Nazar, 1991) were all obtained by PCR according to the published sequences and confirmed by sequencing. For Northern analyses, equal amounts of the total RNAs (Stg) were used for all samples and the probe specific for 18S rRNA was employed to monitor RNA loading.

3. Virus progeny RNA analysis

The viral progeny RNAs were recovered from plants inoculated with each of the recombinant TMV transcripts and analyzed by RT-PCR and DNA sequencing. The cucumoviral 2b coding sequences cloned at the PmeI site of pTMV-30B were first amplified by RT-PCR using a pair of primers flanking the PmeI site of pTMV-30B. The amplified fragments were then purified from agarose gels and either sequenced directly using the same pair of primers or cloned into a plasmid vector before sequencing.

Example 8

1. Induction of hypersensitive cell death

N. tabacum cv. Samsun (nn) does not contain the N gene and is thus susceptible to infection by many TMV strains, including U1 and U5 (Mathews, 1991). Systemically infected plants show characteristic leaf mosaic symptoms. The vector pTMV-30B (Fig. 4) is similar to the TMV-based *in planta* expression vectors previously described (Donson et al., 1991; Kumagai et al., 1995), except that expression of the inserted foreign gene was driven by the 22 U1 coat protein (CP) subgenomic RNA (sgRNA) promoter (arrow 2, Fig. 4) whereas the CP gene and its sgRNA promoter (arrow 3, Fig. 1) were obtained from the U5 strain (W.O. Dawson, unpublished data). The recombinant viral RNA transcribed from pTMV-30B was named TMV-30B. This nomenclature system is used throughout the text. TMV-30B infected Samsun plants displayed a milder systemic mosaic than did Samsun plants infected with the U1 strain of TMV.

The coding sequences of Tav2b and Cmv2b were cloned downstream of the U1 sgRNA promoter of pTMV-30B to give pTMV-T2b and pTMV-C2b (Fig. 4), respectively. Local necrotic lesions, a morphological marker of the HR, appeared on the tobacco leaf inoculated with TMV-T2b about three days post-inoculation (dpi) whereas the rest of the plant remained symptomless for as long as observations were made (five weeks). In contrast, TMV-C2b did not induce necrotic lesions on the inoculated leaf, and produced mosaic symptoms on the upper uninoculated systemic leaves. These latter symptoms were similar to those caused by infection with TMV-30B.

To determine the distribution pattern of TMV-T2b and TMV-C2b in the inoculated tobacco plants, total RNAs were extracted from the inoculated and systemic

leaves and analyzed by Northern blot hybridization using a probe specific to the genomic RNA of TMV (indicated on the right of Fig. 5). Similarly high levels of accumulation of genomic RNAs were detected in both the inoculated leaves (top panel) and the systemic leaves (middle panel) of the plants that were inoculated with either TMV-30B (lane TMV) or TMV-C2b (lane C2b). In comparison, a much lower level of TMV-T2b accumulated in the inoculated leaves (lane T2b, top panel) and no accumulation was detected in the systemic leaves (lane T2b, middle panel). The presence of the coding sequence of Tav2b or Cmv2b in the progeny viral RNAs extracted from the inoculated leaves was confirmed using sequence-specific probes and by sequencing the cDNA fragments obtained from reverse transcription - polymerase chain reaction (RT-PCR). In addition, the genomic RNAs of TMV-C2b and TMV-T2b migrated more slowly than the corresponding genomic RNA of TMV-30B during denaturing agarose gel electrophoresis (Fig. 5, compare lane TMV with the four lanes next to it). Thus, the expression of Tav2b induces hypersensitive cell death and strong virus resistance in the Samsun tobacco cultivar. In contrast, Cmv2b is inactive in this manner.

2. <u>PR gene expression and virus resistance by Tav2b</u>

To test whether or not the challenge inoculation of TMV-T2b also lead to transcriptional induction of genes encoding the PR proteins - molecular markers associated with disease resistance responses, total RNAs extracted from the tobacco leaves at different time points following TMV-T2b inoculation were subjected to Northern blot analysis using probes specific for PR-1a mRNA or PR-5 mRNA. As shown in Fig. 6, the synthesis of both PR-1a (top panel) and PR-5

(lower panel) mRNAs was detectable three dpi and reached maximums of steady-state RNA at five (PR-1a) and seven (PR-5) dpi, respectively. The timing of mRNA induction at three dpi coincided with the appearance of macroscopic necrotic lesions. In contrast, the transcription of PR-1a mRNA (Fig. 5, lower panel) and PR-5 mRNA was not significantly induced in plants infected by TMV-30B (Fig. 5, lane TMV) or TMV-C2b (Fig. 5, lane C2b) even at ten dpi (or at any other earlier time points). Thus, the challenge inoculation by TMV-T2b also led to the transcriptional induction of PR-1a and PR-5 genes. The hypersensitive cell death, PR gene expression and strong virus resistance induced by Tav2b in the Samsun cultivar are the typical host responses associated with the gene-for-gene disease resistance mechanism. Thus, these results indicate that Tav2b, when expressed from the TMV genome, functions as an Avr gene.

Example 9

(Full-Length Sequence Requirement for Avr Activity)

Point mutations were introduced into the coding sequence of Tav2b in the plasmid pTMV-T2b to give pTMV-TΔ2b1 and pTMV-TΔ2b2 (Fig. 4). The recombinant virus TMV-TΔ2b2 was not expected to yield any Tav2b product in infected plants because the second codon (GCA) was converted to a stop codon (TAA). In pTMV-TΔ2b1 both codons 44 (CAA) and 46 (CGA) of Tav2b were changed to stop codons (TAA and TGA). Neither TMV-TΔ2b1 nor TMV-TΔ2b2 induced the formation of necrotic lesions in the inoculated leaves and the inoculated plants developed systemic mosaic symptoms similar to those caused by TMV-C2b. Both TMV-TΔ2b1 and TMV-TΔ2b2 accumulated to detectable levels in the

inoculated leaves and systemic leaves, as revealed by Northern blot hybridization (Fig. 5, top and middle panels, lanes TΔ2b1 and TΔ2b2). Progeny analysis by RT-PCR and sequencing showed that the introduced mutations in both TMV-TΔ2b1 and TMV-TΔ2b2 were stably maintained and no second-site mutations were detected in the inserted TAV sequence. Furthermore, unlike TMV-T2b, infection by TMV-TΔ2b1 or TMV-TΔ2b2 did not lead to the transcriptional induction of PR-1a (Fig. 5, bottom panel, lanes TΔ2b1 and TΔ2b2; Fig. 6, left four lanes) or PR-5 (data not shown) genes. Thus, the induction of hypersensitive cell death, PR gene expression and strong virus resistance in the Samsun cultivar by TMV-T2b correlates with the ability of TMV-T2b to encode the full-length translatable Tav2b open reading frame. This suggests that the encoded protein, Tav2b, is the active molecule and the observed Avr activity is not related to the inserted TAV RNA sequence. This suggestion is also supported by a loss of the Avr activity observed for a mutant form of Tav2b, T2bC (see below), which contained nucleotide substitutions introduced at positions different to those occurred in TΔ2b1 and TΔ2b2. Furthermore, as TMV-TΔ2b1 infected the Samsun cultivar systemically, expression of the truncated Tav2b of 44 amino acids (aa) must be insufficient to elicit the resistance responses.

Example 10

(Functional domains of the Tav2b protein)

The above experiments of Examples 7 & 8 indicated that Tav2b, when expressed *in cis* from the TMV vector, induced hypersensitive cell death and strong virus resistance in the Samsun (nn) tobacco cultivar.

However, Cmv2b is not active in this manner when similarly expressed from the TMV genome. Cmv2b and Tav2b are encoded by two different virus species from the same *Cucumovirus* genus (Ding et al., 1994; Shi et al., 1997). The predicted amino acid sequences of the two proteins are 24% identical (46.2% similarity), the least conserved pair among the known cucumoviral 2b proteins (Ding et al., 1994). As the Tav2b protein is only 95 aa long, results from deletion mutants such as TMV-TΔ2b1 may not be informative in mapping functional domains of Tav2b. Thus, Tav2b as encoded by pTMV-T2b was progressively replaced from either the N-terminus (pTMV-CT1, 2, and 3) or the C-terminus (pTMV-TC1, 2, and 3) by the equivalent regions of Cmv2b according to the alignment of the known cucumoviral 2b proteins (Ding et al., 1994). The structures of the resultant 2b chimeras are given in Fig. 3. Regions (shown as numbers of aa) derived from Tav2b are depicted as open sections and those from Cmv2b depicted as filled sections.

Results of the infectivity experiments using the recombinant TMV transcripts from the six chimaeric 2b constructs are shown in Fig. 7 and summarized in Fig. 3. CT1, CT2 and CT3 resulted from the replacements of the N-terminal 91, 69 or 50 aa of Tav2b by the equivalent regions of Cmv2b (Fig. 3). The tobacco plants were susceptible to all three of the TMV recombinants in which the N-terminal 50 aa or more of the encoded 2b protein were derived from Cmv2b (TMV-CT1, 2, and 3). None of these recombinants induced necrotic lesions (data not shown) and all accumulated in both the inoculated and systemic leaves (Fig. 7, lanes CT1, CT2 and CT3). The infected plants also displayed systemic mild mosaic symptoms similar to those caused by TMV-C2b. Thus, absence of

the Tav2b N-terminal 50 aa in a 2b chimera resulted in the loss of the avirulence function, indicating that this region is essential for the induction of virus resistance and hypersensitive cell death in the host.

Consistent with the above observation, the data obtained from three TMV-TC recombinants further indicated that the Tav2b N-terminal sequence of 69 aa was sufficient to confer virus resistance in cv. Samsun. TC1, TC2 and TC3 resulted from the replacements of the C-terminal 4, 26 or 50 aa of Tav2b by the equivalent regions from Cmv2b (Fig. 3). The N-terminal 69 and 91 aa respectively of TC1 and TC2 were derived from Tav2b (Fig. 3). Both induced strong virus resistance in the challenged tobacco plants. This is because both TMV-TC1 and TMV-TC2 accumulated to very low levels in the inoculated leaves (Fig. 7, lanes TC1 and TC2 of the upper panel) and no accumulation of viral RNAs was detected in the systemic leaves (lanes TC1 and TC2 of the lower panel). The challenged plants remained symptomless for as long as observations were made (five weeks). Reprobing the filter shown at the upper panel of Fig. 7 with the PR-1a probe showed that transcription of PR-1a mRNA was induced in the tobacco leaves inoculated with TMV-TC1 and TMV-TC2 but not in those inoculated with the remaining four viruses. Thus, the function of the N terminal 69 aa of Tav2b in resistance activation is independent of its remaining 26 amino acids at the C-terminus.

TC3 contained 50 aa from the N-terminus of the Tav2b protein (Fig. 3) and did not induce virus resistance because TMV-TC3, like TMV-CT3, infected the tobacco plants systemically (Fig. 7, bottom panel). This result is consistent with the finding of Example 9 that TΔ2b1, encoding a truncated Tav2b of 44 aa, did not induce virus resistance in the tobacco plants. The

fact that TC2, but not TC3, is avirulent (Fig. 3) suggests that amino acids 50 - 69 of Tav2b play a key role in the resistance activation. However, CT3 contained the same 20 aa from Tav2b, but it failed to induce virus resistance (Fig. 7), indicating that presence in a 2b chimera of this region of Tav2b, unlike the more extended N-terminal region of 69 aa, is not sufficient to activate virus resistance.

Although the Samsun cultivar is resistant to both TMV-TC1 and TMV-TC2, TMV-TC1 induced necrotic lesions in the inoculated leaves identical to those triggered by TMV-T2b. Under the same conditions, TMV-TC2 caused no visible hypersensitive cell death (Fig. 3). This result, a local and symptomless infection by TMV-TC2, was consistent and reproducible in six independent experiments, involving a total of 37 plants. This indicates that the sequence encompassing amino acids 70-91 of Tav2b is required for triggering hypersensitive cell death. However, TMV-CT2 and TMV-CT3, both of which encode the same 22 amino acids from Tav2b, did not cause necrotic lesions (Fig. 3), suggesting that this sequence alone is insufficient to trigger hypersensitive cell death.

Example 11

(Abolishment of Tav2d avirulance activity)

To further analyze the avirulence determinant, ^{21}Lys and ^{27}Arg, both positively charged and located within the resistance domain of the avirulent Tav2b, were replaced with ^{21}Val and ^{27}Ser found at the aligned positions of the virulent Cmv2b (Ding et al., 1994). TMV-T2bC, expressing the resultant Tav2b mutant, did not elicit any of the disease resistance responses associated with wild type Tav2b. Neither necrotic

lesions nor transcriptional induction of PR 1a mRNA were observed in the inoculated Samsun leaves. Furthermore, the TMV-T2bC inoculated plants became systemically infected. Northern blot analysis showed that TMV-T2bC accumulated to similar levels to TMV-TΔ2b2 in both the inoculated and the systemic leaves of the infected plants. RT-PCR and DNA sequencing showed that the introduced mutations were maintained in the progeny viral genome and no additional mutations were found in the Tav2b coding sequence of TMV-T2bC. Thus, the two aa substitutions abolished the Avr activity of Tav2b in this tobacco cultivar.

Example 12

(Tav2b activity in *N. benthamiana*)

We have established previously that Cmv2b expressed from a potato virus X (PVX) vector functions as a virulent determinant and suppresses PTGS of a green fluorescent protein (GFP) transgene in *N. benthamiana* plants (Brigneti et al., 1998). *N. benthamiana* plants were also fully susceptible to TMV-T2b. Infected plants displayed a systemic necrosis and began to collapse at 14 dpi and died a few days after. TMV-C2b induced a much milder systemic infection that did not resulted in the death of the infected plants; this difference in virulence between Tav2b and Cmv2b has been observed previously (Ding et al., 1996). Plants systemically infected with TMV-TΔ2b2 showed a mild chlorosis similar to that caused by TMV-30B infection. Accumulation of TMV-T2b and TMV-TΔ2b2 in both the inoculated and systemic leaves were confirmed by Northern blot hybridization.

Thus, Tav2b, like Cmv2b, was not recognized as an Avr gene in *N. benthamiana*.

To test the role of Tav2b in suppressing PTGS, the TAV sequences from pTMV-T2b and pTMV-TΔ2b2 were transferred into a PVX vector (pP2C2S) to give pPVX-T2b and pPVX-TA2b2, from which RNA transcripts were obtained and inoculated to *N. benthamiana* plants exhibiting complete systemic PTGS of the GFP transgene. PVX-T2b was similarly virulent to TMV-T2b in *N. benthamiana* and PVX-T2b infection led to a rapid death of the infected plants. However, at 12 dpi when the severe systemic necrosis had not developed, the newly emerging leaves of PVX-T2b-infected plants were green fluorescent under UV illumination, whereas old leaves that developed prior to virus infection remained red fluorescent. Northern analysis of RNA extracted at 12 dpi showed a significant level of GFP RNA accumulation in these green fluorescent leaves. By comparison, at 12 dpi, PVX-C2b-infected plants remained red fluorescent and had 1 GFP RNA levels that were below the limit of detection although the newly emerging leaves of those plants became green fluorescent and accumulated high levels of GFP RNA a few days after (Brigneti et al., 1998). As expected, PVX-TΔ2b2 was much less virulent than PVX-T2b in *N. benthamiana* and no suppression of PTGS of the GFP transgene was observed in PVX-TΔ2b2-infected plants at 18 dpi or at 35 dpi. Thus, Tav2b is a virulent determinant and a suppressor of PTGS in *N. benthamiana*.

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